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(64) Fibronectins.

(67) The polypeptide sequence of the collagen-binding and fibrin-binding parts of fibronectin and the corresponding cDNA sequences have been determined.

The collagen-binding polypeptide is useful in methods of purification and the fibrin-binding polypeptide is useful for targetting therapeutic substances on natural fibrin.

FIBRONECTINS

Fibronectins (FNs) constitute a class of high molecular weight glyco-proteins that have a key role in various contact processes of the vertebrates such as cell attachment and spreading, cell migration, control of cell morphology, differentiation and oncogenic transformation. All these biological activities imply interaction of FN with cells and with extracellular materials. Binding activities for collagen, heparin, fibrin, cell surfaces, bacteria and DNA have been located in different domains of the FN molecule (for review, see Yamada, 1983).

FN is one of the most versatile known proteins, both functionally and structurally. FN molecules are usually dimers of similar but not identical polypeptides of MW \approx 250,000. Cellular FN is found in a fibrillar component of the extracellular matrix of fibroblasts and other cell types. Plasma FN is a soluble molecule present in high concentrations in plasma (300 μ g/ml) and probably involved in opsonization, wound healing and haemostasis (Yamada, 1983; Hynes & Yamada, 1982). Partial primary structure data have revealed high conserved amino acid sequences both between the two FN forms and among FNs from different species: bovine plasma (Petersen *et al.*, 1983), bovine cellular (Kornblihtt *et al.*, 1983), human plasma (Pande & Shively 1982; Garcia-Pardo *et al.*, 1983), human cellular (Kornblihtt *et al.*, 1983, 1984a; Oldberg *et al.*, 1983), rat plasma (Schwarzbauer *et al.*, 1983). These data have tended to confirm that the basic FN polypeptide contains three different types of internal repeats (homology types I, II and III, approximately 40, 60 and 90 amino acids long respectively, as originally shown in bovine plasma FN (Skorstengaard *et al.*, 1982; Petersen *et al.*, 1983). Variations on this basic fibronectin structure account for the differences between cellular and plasma fibronectins and also between the polypeptide chains of both forms.

The diverse forms of fibronectin seem to be generated by transcription of a single gene into a common precursor which undergoes alternative splicing (Vibe-Pedersen et al., 1984). To-date, at least two regions have been described
5 where this type of variation occurs. In certain human cell lines (fibroblasts, Hs578T) FN mRNAs can be distinguished by a 270 nucleotide segment (ED) that encodes exactly one of the homology type III. This ED segment seems to be absent in the liver hepatocyte mRNAs which are
10 the source of plasma FN (Kornblihtt et al., 1983, 1984b). Schwarzbauer et al. (1983) have reported three different FN mRNAs arising by alternative splicing in rat liver which differ in an area (IIICS) located to the 3' carboxy terminus side in the protein of the ED region. The
15 difference sequence does not belong to any of the known internal homologies and it is inserted between the last two type III homology repeats, near the COOH terminus. In addition, Umezawa et al. (1985) have reported further variations in the equivalent IIICS area of human liver
20 FN mRNA, bringing the total to 5 alternative motifs for this area. The differences observed between FN polypeptides are thus the consequence of internal primary sequence variability (Kornblihtt et al., 1984a 1984b; Schwarzbauer et al., 1983), due to alternative splicing in at least two
25 distinctive regions of the pre-mRNA (Tamkun et al., 1984; Vibe-Pedersen et al., 1984; Umezawa et al., 1985).

The complete amino acid sequence of mature human FN polypeptides has now been determined from the nucleotide sequence of multiple cDNA clones as described below. The
30 polypeptide length varies from 2146 to 2325 amino acids, depending on which internal alternative splicing has taken place.

The present invention thus makes it possible to provide any desired part of the fibronectin molecule and
35 in particular polypeptides having each of the separate binding activities of fibronectin separate from the others.

In the accompanying Figure 2, the binding sites of each part of the FN amino-acid sequence are given. While some of these were previously known, the sequence for the human collagen-binding site (lines 8-13 in the Figure) is new. Lines 9 and 10, involving the type II homology, are believed to be of particular significance, and to incorporate most or all of the collagen-binding ability.

The present invention thus provides novel polypeptides having substantially the amino acid sequence from 277 to 577 shown in Figure 2, or any continuous part thereof having collagen-binding activity, and especially the sequence from 379 to 445. In practice such polypeptides may be linked to further amino-acid residues not affecting the desired end use of the collagen-binding polypeptide, including additional 15 residues of the fibronectin molecule itself.

Similarly other sequences of the fibronectin molecule may be utilized for their ability to bind to other molecules. Thus, as shown in Figure 2, the polypeptide sequence from 21 to 241 is associated with binding to fibrin, heparin and 20 Staphylococcus aureus. Other sequences are, as shown, associated with binding to DNA, cells and alternative heparin and fibrin binding sites.

The novel polypeptides may be made by culturing cells containing endogenous DNA coding for the polypeptide and 25 separating the polypeptide from the metabolic products. Thus, the appropriate DNA sequences may be cloned into a competent strain of E.coli or other microorganism, e.g. a yeast such as Saccharomyces cerevisiae, the latter cultivated, and then the desired polypeptide isolated from 30 the cultivation products. Figure 3 shows the complete DNA sequence for fibronectin and the associated amino-acid residues, and from this the cDNA sequence required for cloning the expression of any desired part of the fibronectin molecule may be easily determined.

35 The DNA sequence coding for the collagen-binding polypeptide runs from coordinate 1147 to coordinate 1351, and the sequence coding for the fibrin-binding polypeptide runs from coordinate 73 to 738.

More particularly, however, in accordance with the present invention,

the clones herein described as pFH54, pFH134, pFH16 and pFH6 and similar clones may be used to produce corresponding polypeptides by expression in E. coli or other appropriate microorganisms. pFH134 and pFH16 contain the DNA sequence for the collagen-binding part of the fibronectin molecule and may be used to generate a polypeptide having the collagen-binding activity of fibronectin without its other binding affinities. pFH6 can be used to transform competent E. coli for the expression of a polypeptide binding fibrin and heparin.

The manner in which the clones pFH54, pFH134, pFH16 and pFH6 were obtained is described below, and repetition of the methods referred to will give similar clones with essentially the same or only slightly different utility. It will be appreciated in this connection that the isolation of useful cDNA sequences using the total cellular RNA from an appropriate source, i.e. cells naturally capable of expressing the desired protein or polypeptide, is a matter of routine experimentation for the person skilled in the art using the currently available techniques, particularly when, as is the case here, the actual amino acid sequence for the desired protein or polypeptide and the corresponding DNA sequence are both known. The experimental section below mentions appropriate techniques which have been found to be effective but it is to be expected that other known techniques would be equally applicable. Similarly choice of appropriate sources of RNA, vectors, and competent microorganisms for transformation from the many materials which are now available to the experimentalist is well within the ordinary skill in this art.

It will be appreciated that when a desired amino-acid sequence of fibronectin is expressed by a transformed microorganism it may be associated with a polypeptide characteristic of the microorganism itself. This may be immaterial to the intended use of the polypeptide but, in some cases, eg. if the polypeptide is to be used in therapy,

the presence of the additional amino-acid residues may be unacceptable. In that case the polypeptide must be subjected to an additional treatment, eg. with a protease, to separate the desired polypeptide free from undesired additional amino-acid residues.

As indicated above, the present invention is of especial interest in that it provides a means for transforming an appropriate microorganism to make it capable of expressing a polypeptide able to bind to collagen and/or fibrin. A polypeptide able to bind to collagen can be used, for example, to facilitate affinity purification of valuable polypeptides. Thus if the collagen-binding polypeptide is expressed in a form in which it is bound to another polypeptide of interest or if it is linked, after isolation, to such a polypeptide, the combined polypeptides may be purified by affinity chromatography on a column of bound gelatin (i.e. collagen), and then, after the purification, the desired polypeptide may be separated, eg. by an enzymatic hydrolysis, from the collagen-binding polypeptide.

A polypeptide able to bind to fibrin may be used in therapy to target a therapeutic agent on natural fibrin, eg. a blood clot. For example, a fibrinolytic enzyme bound to the polypeptide would have improved clot-dissolving properties, since it would have improved adhesion to its target.

In the accompanying drawings, Fig. 1 shows a restriction enzyme map of seven cDNA clones covering 76% nucleotides from the poly(A) tail of human FN mRNA. Human FN mRNA has been estimated to be 7900 nucleotides long (Kornblihtt et al., 1983). The clones cover the complete coding region for the mature protein (bottom diagram showing binding sites) and the 3' non-coding region. The dotted lines indicate segments that are absent from the corresponding cDNA clones but that must have been

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synthesized in the first strand cDNA reaction and that were lost as a result of the failure of the Klenow enzyme to complete the second cDNA strand. Numbering on the map is in base pairs.

- 5 Fig. 2 shows the complete amino acid sequence of a human FN polypeptide. Residues 1 and 2325 are the NH₂ and COOH termini of the mature protein. The sequence was deduced from the nucleotide sequence (see Figure 3) of the cDNA clones depicted in Fig. 1. Alignment shows
- 10 internal homologies. Gaps were introduced to maximize the homology. Identical residues within a type of homology are boxed. The cell recognition tetrapeptide RGDS (Pierschbacher & Ruoslahti, 1984) is underlined. Positions 17 (Ser), 21 (Cys) and 42 (Val) are reported as Cys, Ser
- 15 and Ala respectively by Garcia-Pardo et al (1983). The FN polypeptide shown in this Figure has 2325 residues with a MW = 255,905. If the mass contributed by the carbohydrate side chains, estimated to be 9% of the protein mass (Yamada, 1983), is added, the molecular weight of this
- 20 FN polypeptide would increase to approximately 279,000. This figure appears to be considerably higher than the weights of the FN monomers estimated by SDS-PAGE (230-250,000). The discrepancy could be explained by the poor resolution of the SDS gels in the range of high molecular weight proteins
- 25 together with the lack of appropriate protein standards in that range.

Symbols are as follows: ■, free SH groups; λ, sites for carbohydrate side chains; Δ, cleavage site for chymotrypsin. ▲, cleavage site for plasmin. The multiple

30 fibronectin polypeptides can be generated by all the possible permutations of the alternative splice regions in lines 26 and 30 (as explained below).

- Fig. 3 shows the complete nucleotide sequence for the human FN polypeptide of Figure 2 deduced from the
- 35 sequences of the cDNA clones of Figure 1.

Fig. 4 shows the variations of the FN primary structure.

the complete structure of the mature protein. Black boxes are type I homologies; dashed boxes, type II homologies;

empty boxes, type III homologies.

B and C show schematic representation of the different FN
5 polypeptides that could arise by the translation of the
multiple FN RNAs observed in the ED region (B) and in the
IIICS region (C). The name of the cDNA clones representing
the corresponding encoding mRNA species is shown to the right
of each polypeptide. ^ indicates contiguity. λ rlf2, 4 and 6
10 were isolated from a rat liver cDNA library (Schwarzbauer
et al, 1983), pFHL1 and 8 from a human liver cDNA library,
and pFHL was isolated from the Hs578T cell line cDNA library
(Kornblihtt et al, 1983). It will be noted that all the
variations include the zones binding to collagen and fibrin.

15 Figure 5 shows, in more detail than Figure 1, part of the
fibronectin protein indicating the position of the collagen-
binding domain and internal homologies (I, II, and III).
Below, the position and sizes (in base pairs) of the series of
cDNAs used in the bacterial expression experiments described
20 below is indicated. Only the restriction enzyme sites
relevant to the cDNAs pXFN1-8 are shown. The flanking Hind
III and Bam HI sites of pFH134 and pFH16 occur in the
polylinker of the vector.

Figure 1 shows the restriction map of different cDNA
25 clones covering the 3' non-coding region and the complete
coding region for the mature protein of human FN mRNA. The
isolation of clones pFH1, pFH111 and pFH154 has been
previously described (Kornblihtt et al, 1983, 1984a) and
nucleotide sequence and deduced amino acid sequence of the
30 latter has been published before (Kornblihtt et al, 1984b).
The clones pFH54, pFH134, pFH16 and pFH6 are new. Isolation
of these four cDNA clones covering the 5' third of the map
involved the synthesis of an oligonucleotide primer. The
sequence (namely 5-GCTGAACCATTTGCTGAGC) of the primer was
35 complementary to the mRNA sequence of a region close to the
5' end of clone pFH154. The oligonucleotide was used to prime
reverse transcription of total RNA from Hs578T cells (Hackett
et al, 1977) and a cDNA library was prepared as described
below. The clones pFH54, pFH134, pFH6 and pFH16 were selected
for further analysis. The complete nucleotide sequence of
these clones was determined and comprised 7692 bp, of which

6972 bp correspond to the coding region and 720 bp to the 3' nontranslatable region and poly(A)tail. The sequence is included in the full DNA sequence for FN shown in Figure 3.

The amino acid sequence of human fibronectin deduced from the nucleotide sequence of the clones in Figure 1 is shown in Figure 2. The alignment in Figure 2
 5 maximises internal homologies. The complete FN chain presents regions which have 3 different types of internal homologies (types I, II and III) (Petersen et al, 1983) and regions which have no homologous counterparts within the molecule. The latter are the NH₂-terminal and COOH-
 10 terminal segments and the internal connecting strands. From NH₂-to COOH-terminus, FN is formed by one 20-residue long NH₂-terminal segment (Fig. 2, line 1), five units of type I homology or fingers (lines 2 to 6), one connecting strand
 (line 7), one finger (line 8), two units of type II
 15 homology (lines 9 and 10), three fingers (line 11, 12 and 13), one unit of type III homology (line 14), one connecting strand (line 15), fourteen units of type III homology (lines 16 to 29, including the ED polypeptide), one
 connecting strand (IIICS, line 30), one unit of type III
 20 homology (line 31), one connecting strand (line 32), three fingers (lines 33, 34 and 35) and the COOH- terminal segment (line 36).

The primary structure of FN reflects a level of order and complexity not seen before in any other protein. The
 25 symmetry in the array of the 16 units of type III homology is particularly interesting. Two type III units (Fig. 2, lines 14 and 31) are separated by connecting strands (lines 15 and 30) from a central block, having the remaining fourteen in a juxtaposed way. The degree of
 30 homology within the type III units is very high. Three residues are conserved in all the units, viz Trp (Fig. 2, box with residue 599 at the top), Leu (box with residue 640 at the top) and Tyr (box with residue 646 at the top). The conserved residues are distributed in two peaks around
 35 the Trp and the Tyr, separated by a valley of non homology. is believed that the degree of order and conservation in

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the type III sequences must reflect particular constraints of the secondary structure of the central region of FN. This region is not stabilized by disulphide bridges since the only two Cys residues present in the type III sequences (positions 1201 and 2075 in Figure 2) have been shown to exist in a reduced form. (Vibe-Pedersen et al., 1982; Smith et al., 1982).

Several binding activities have been assigned to different regions of the FN molecule (see Figs 1 and 2). However, only in the case of the ability to bind cells, has the actual binding site been identified so far. In fact, Pierschbacher and Ruoslahti (1984) demonstrated that the tetrapeptide Arg-Gly-Asp-Ser (RGDS) is responsible for the cell attachment activity of FN. This tetrapeptide is present only once in the sequence of Fig. 2 at positions 1493 to 1496, within one of the type III units. Fig. 2 also shows that the optimal alignment of the type III sequences in this area is obtained only if the tetrapeptide is considered to be an extra element, allowing four gaps in the corresponding regions of the rest of the type III units. It is probable that, as well as the cell binding site, other binding sites or biological activities within type III sequences reside in non-conserved stretches. The tetrapeptide has also been found in other proteins (Pierschbacher & Ruoslahti, 1984) including the α chain of fibrinogen which shows cell attachment activity.

An important feature of FN gene expression is the generation of slightly different polypeptides by differential processing of the common mRNA precursor (Vibe-Pedersen et al., 1984). Figure 4A shows diagrammatically the localization of the two regions of variability observed so far along the FN molecule (Schwarzbauer et al., 1983; Kornblihtt et al., 1984a 1984b). Figures 4B and 4C show the types of polypeptide that can arise from the translation of the different mRNAs generated in the ED (Fig. 2 line 26) and IIC5 (Fig. 2, line 30) regions respectively. This diagram combines observations made on

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both human and rat fibronectin. At least 10 different FN polypeptides can be generated from a single gene if it be assumed that all the permutations between the ED and IIIICS segments are possible. This is consistent with the

5 FN polypeptide heterogeneity observed in the bidimensional gel electrophoresis analysis of cellular and plasma FNs found in vivo. Homo- or hetero- dimeric FN molecules can then be formed from the FN polypeptide pool. The biological significance of this complex situation is not

10 yet clear. However, it is to be noted that the ED and IIIICS variable regions are intercalated between the cell-heparin and heparin-fibrin binding sites. The distance between these biologically active sites of the molecule may be critical for the FN function. For example, plasma

15 FN is 1 to 2 orders of magnitude less active than cellular fibronectin in restoring morphology and alignment to a transformed fibroblast cell line (Yamada & Kennedy, 1979). Further the mRNAs carrying the ED segment are present in fibroblasts (one source of cellular FN) but not in liver

20 cells (one source of plasma FN) (Kornblihtt et al, 1984b). It is possible that the function of the ED is to increase the distance between the cell binding tetrapeptide and the heparin binding site, resulting in an enhanced binding activity of the cellular FN molecule.

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EXPERIMENTALRNA Preparation

Human cell line Hs578T (Hackett et al., 1977) was cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum. Total RNA was extracted from confluent cell monolayers by the guanidine-HCl method (Chirgwin et al., 1979). Between 2 and 4 mg of total RNA were extracted from 4×10^8 cells.

Other sources of RNA could be used if preferred, eg. fibroblasts or liver cells.

Isolation of fibronectin cDNA clones

All the cDNA clones depicted in Figure 1 were obtained using Hs578T cell RNA as template. Isolation of clone pFH1 by oligonucleotide probing was described by Kornblihtt et al. (1983). Isolation of clones pFH11 and pFH154 by "mRNA walking" (oligonucleotide priming) was described by Kornblihtt et al. (1984a). This latter procedure was used for the isolation of the new clones pFH54, pFH134, pFH16 and pFH6. An oligonucleotide primer complementary to the mRNA region close to the 5' end of pFH154 was synthesized by the method of Gait et al. (1980). The oligonucleotide was used to prime reverse transcription of total RNA from Hs578T cells (Hackett et al., 1977). Blunt ended ds cDNA was prepared and cloned into the plasmid pAT153/PvuII/8 (Anson et al., 1984) in *E. coli* MC1061 as previously described (Kornblihtt et al., 1983). Colonies were screened using as probe a restriction fragment from the 5' end of pFH154 lacking the primer sequences, labelled by filling in at one end. In this way, clones pFH54 and pFH134 were obtained. In a second step, clones pFH16 and 6 were obtained by screening with an end labelled probe for the 5' end of clone pFH134.

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Restriction fragments of the fibronectin cDNA were filed in with the Klenow fragment of DNA polymerase I and blunt ended ligated into SmaI cut/phosphatased pEX 1, 2 or 3 vector. Transformations were carried out using the *E. coli* strain
5 LK111 (Zabeau et al, 1982) harbouring the plasmid pc1857 which specifies kanamycin resistance and carries the cl857 allele (Remaut et al, 1983). Colonies were transferred to Whatman 541 filter paper (Gergen et al, 1985) and screened with either 3' end labelled (Maxam et al, 1977) or nick
10 translated probes (Rigby et al, 1977).

Sequence determination

Inserts from clones were excised from the vector DNA by digestion with appropriate restriction enzymes, separated in agarose gel electrophoresis, and recovered by electroelution
15 (Girwitz et al, 1980). Most of the sequencing was performed by the chemical degradation procedure of Maxam and Gilbert (1980). Some regions were sequenced by the chain terminator method (Sanger et al, 1977). For that purpose, the relevant fragments were isolated, digested either with AluI or HaeIII
20 and ligated to a SmaI digested M13mp9 vector (Messing & Vieira, 1982), previously treated with calf intestinal phosphatase to prevent its circularization. The ligation mixtures were used to transform competent *E. coli* JM101 and recombinants were selected as clear plaques by insertional
25 inactivation of the β -galactosidase gene (Messing et al, 1981). Single stranded DNA was prepared by standard procedures (Winter & Fields, 1980) and the inserts were sequenced using a "universal" 17-nucleotide long primer (Duckworth et al, 1981).
Preparation of bacterial extracts

30 Bacteria carrying recombinant plasmids were grown at 30°C for 24h and expression of the cro/ β -galactosidase fusion protein induced by shifting to 42°C for 2h. Bacteria were pelleted at 1200 g and washed with 50 mM TrisHCl, pH 7.4, 170 mM NaCl. Cells were resuspended in the same buffer
35 containing lysozyme (2.5 mg/ml) and sonicated for 2 min on ice. The lysate was centrifuged at 45,000 g for 30 min at

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4°C. The pellet was resuspended in 7 M urea in 10 mM Tris HCl, pH 7.4, 1mM EDTA and incubated at room temperature for 30 min. The solubilised extract was dialysed extensively against 50 mM Tris HCl pH 7.4 at 4°C, and then centrifuged
5 at 45,000 g for 30 min at 4°C.

Gelatin-Sepharose chromatography

Gelatin-Sepharose was either obtained from Sigma Chemicals (St. Louis, MO, USA) or prepared by linking gelatin (pig skin type 1, Sigma Chemicals) to CNBr-activated
10 Sepharose CL.4B (Pharmacia, Uppsala, Sweden). Chromatography of bacterial extracts on gelatin-Sepharose was carried out as described by Ruoslahti *et al* (1982). The efficacy of the gelatin-Sepharose matrix was verified using purified human plasma fibronectin (Sigma Chemicals).

15 Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis was carried out in 0.1% (w/v) SDS in Tris/glycine buffer on 7.5% (w/v) acrylamide slab gels (19). Gels were stained with 0.1% Coomassie blue in methanol/water/acetic acid (4:5:1 by vol.).
20 Immunoblotting was performed as described by Towbin *et al*. (1979). Polypeptides, electrophoretically transferred to nitrocellulose were probed with rabbit anti(human plasma fibronectin) serum (1:500 in phosphate buffered saline, 10% newborn calf serum and 0.05% Tween 20). Bound immunoglobulin
25 was visualised using alkaline phosphatase conjugated goat anti-(rabbit IgG) (1:1000; Sigma Chemicals).

Protein assay

Protein was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

30 Construction and characterisation of fibronectin expression plasmids

The human fibronectin cDNA clones, pFH134 and pFH16, encompass all or part of the collagen-binding domain of fibronectin identified by proteolytic cleavage of the protein (see Fig. 1 and Fig. 5). These cDNAs were therefore chosen
35 as the starting point for investigating the expression of a functional collagen-binding site in *E. coli*. The pEX vectors

used for cloning enable exogenous gene sequences to be inserted into a polylinker in all three reading frames at the 3' end of a *cro*-*LacZ* hybrid gene under the control of the λ P_r promoter (Stanley *et al.*, 1984). The 5' ends of the 1.74 kb and 1.04 kb inserts of pFH134 and pFH16 respectively were sequenced (Maxam *et al.*, 1980) to establish the reading frames of the cDNAs and blunt end cloned into the *Sma*I site of pEX2. The recombinant plasmids were introduced into an *E. coli* strain previously transformed with a plasmid encoding the temperature-sensitive λ P_r repressor, c1857. This allows for temperature-inducible expression of the *cro*/ β -galactosidase protein. To test for the production of fibronectin fusion protein by the expression constructs, hybridisation positive clones were grown at 30°C for 24 h and then shifted to 42°C for a further 2 h. Total bacterial lysates were analysed by SDS polyacrylamide gel electrophoresis. Five of ten pXFH134 constructs and one of seven pXFH16 constructs showed the production of high molecular weight polypeptides of sizes consistent with the lengths of the cDNA inserts (~185 kD and ~165 kD respectively). The correct orientation of the fibronectin sequences in pXFH134 and pXFH16 was confirmed by restriction enzyme analyses.

The fusion proteins produced by pXFH134 and pXFH16 accounted for approximately 20% of the total bacterial protein consistent with the previous report for this vector system (Stanley *et al.*, 1984). Both fusion proteins showed some proteolytic degradation, particularly the pXFH134 polypeptide, which appeared to be partially cleaved to the size of the wild-type *cro*/ β -galactosidase (116 kD). Analysis of proteins synthesised over a time course of induction (0 to 120 min) indicated that proteolysis occurred concomitantly with synthesis of the fusion proteins.

The expression of fibronectin antigenic determinants in pXFH134 and pXFH16 was investigated by immunoblotting using a rabbit polyclonal anti-(human plasma fibronectin) serum.

The antiserum reacted with the 185 kD polypeptide synthesised by pXFH134 but not with the pXFH16 fusion protein or the cro/ β -galactosidase polypeptide, indicating that the epitope(s) recognised by the anti-serum lie
5 outside the type II homology units and adjacent type I repeats (Fig. 5). This observation is consistent with the poor antigenicity of the collagen-binding domain of human fibronectin previously reported (Ruoslahti *et al.*, 1979) and
10 most probably reflects the very high level of amino acid conservation in this region (Fig. 2).

Gelatin-Sepharose affinity chromatography

Over-production of β -galactosidase fusions in *E.coli* results in the precipitation of the protein in the cells as insoluble inclusion bodies (Williams *et al.*, 1982, Cheng 1983,
15 Stanley 1983). Thus, when bacteria expressing the pXFH134 plasmid were lysed by sonication and centrifuged, the fibronectin fusion protein was found exclusively in the insoluble pellet. This fraction represented approximately 50% of the total protein of the bacterial lysate. Solubilisation of this material required treatment with 7 M urea and,
20 following dialysis, 60% of the protein remained in solution. This fraction, which was highly-enriched in the fusion protein, was applied directly to a 5 ml gelatin-Sepharose column equilibrated in 50 mM Tris HCl, pH 7.4. The column
25 was washed with 0.5 M NaCl in 50 mM Tris HCl, pH 7.4 until the E_{280} of the flowthrough was <0.01. The cro/ β -galactosidase-fibronectin hybrid protein was eluted from the column as a single symmetrical peak with 4 M urea in the same buffer. Under these conditions fibronectin is specifically released
30 from gelatin-Sepharose (Ruoslahti *et al.*, 1982). In the control experiment using pEX2 only, no binding of the wild-type cro/ β -galactosidase protein was observed.

A functional collagen-binding site has therefore been reconstituted in the pXFH134 fusion protein. It must be
35 noted, however, that the fusion protein specifically eluted from the column represented < 5% of the fusion protein

applied to the column. Thus, not surprisingly, considerable activity is lost due to the insolubilisation of the fusion proteins in the bacterial cells, and subsequent vigorous treatment required to resolubilise them.

- 5 The fibronectin fusion protein produced by pXFH16 was also tested for gelatin-binding and showed similar activity to pXFH134. This indicated that the collagen-binding region occurred within the domain defined at the protein level and strongly implicated the two type II and adjacent type I
10 homology units (see Fig. 5). To further localise the binding site, a series of overlapping expression constructs was made from pFH16 (Fig. 5) and systematically assayed for gelatin-binding activity. The results are summarised in Table 1, and show the consistent involvement of the type II homology
15 units (pXFN 2, 3 and 6).

Table 1

pEX construct	Binding to Gelatin-Sepharose
pXFH134	+
pXFH16	+
pXFN1	-
2	+
3	+
4	-
5	-
6	+
7	-
8	-
pEX2 vector only	-

The binding activity of pXFN134 is almost entirely accounted for by a construct consisting of the two type II homology units (pXFN3). By comparing the gelatin-binding activity of pXFN3 and pXFN6 (both active) with pXFN5 and pXFN8 (both inactive), it may be deduced that the amino acid sequence critical for binding lies in the C-terminal half of the fibronectin fragment in pXFN3, and more particularly from the HinfI site (coordinate 1147 of Figure 3) to the RsaI site (coordinate 1351 of Figure 3). This 66 amino acid sequence represents almost the entire second type II homology unit of fibronectin plus a few amino acids of the adjacent type I homology unit (see Fig. 2).

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- 20 -

CLAIMS

1. A polypeptide sequence having specific affinity for collagen and/or fibrin and containing amino-acid residues in substantially the same order as in the amino-acid residues in the collagen binding and/or fibrin binding part of fibronectin.
2. A polypeptide according to claim 1 comprising at least the collagen-binding portion of the amino-acid sequence 277 to 577 shown in Figure 2.
3. A polypeptide according to claim 1 comprising at least the collagen-binding amino-acid sequence 379 to 445 shown in Figure 2.
4. A polypeptide according to claim 1 comprising at least the fibrin-binding portion of the amino-acid sequence 21 to 241 shown in Figure 2.
5. A polypeptide according to any of claims 1 to 4 bound to a polypeptide sequence not present in fibronectin.
6. Method of purifying a substance which comprises contacting a conjugate of that substance and the collagen-binding portion of fibronectin with immobilized collagen so that the said conjugate binds to the said collagen, and then eluting the said conjugate.
7. Method according to claim 6 in which the said eluted conjugate is then split to remove the said collagen-binding portion, and the said substance is then isolated.
8. A polypeptide according to claim 1 or 4 comprising a fibrin-binding amino-acid sequence bound to a therapeutic agent.
9. A cDNA sequence coding for the polypeptide sequence of fibronectin having specific affinity for collagen and/or fibrin.
10. A cDNA sequence according to claim 9 having the structure shown at 1147 to 1351 in Figure 3b.
11. A cDNA sequence according to claim 9 having the structure shown at 73 to 738 in Figure 3a.
12. A plasmid or other vector containing a cDNA sequence

- 21 -

as defined in any of claims 9 to 11.

13. A microorganism modified by inclusion of a vector as claimed in claim 12.

14. Escherichia coli modified by inclusion of a vector as claimed in claim 12.

- 20 -

CLAIMS FOR DESIGNATED STATE AT

1. Process for producing a polypeptide sequence having specific affinity for collagen and/or fibrin and containing amino-acid residues in substantially the same order as in the amino-acid residues in the collagen binding and/or fibrin binding part of fibronectin, which comprises
5 culturing a cell containing an exogenous DNA sequence coding for said polypeptide sequence, and separating said polypeptide sequence from the metabolic products of said cell.
- 10 2. A process according to Claim 1 in which the said polypeptide comprises at least the collagen-binding portion of the amino-acid sequence 277 to 577 shown in Figure 2.
3. A process according to Claim 1 in which the said polypeptide sequence comprises at least the collagen-
15 binding amino-acid sequence 379 to 445 shown in Figure 2.
4. A process according to Claim 1 in which the said polypeptide sequence comprises at least the fibrin-binding portion of the amino-acid sequence 21 to 241 shown in
Figure 2.
- 20 5. A process according to Claim 3 in which the exogenous DNA has the structure shown at 1147 to 1351 in Figure 3b.
6. A process according to Claim 4 in which the exogenous DNA has the structure shown at 73 to 738 in
25 Figure 3a.
7. Method of purifying a substance which comprises contacting a conjugate of that substance and the collagen-binding portion of fibronectin with immobilized collagen so that the said conjugate binds to the said collagen, and
30 then eluting the said conjugate.
8. Method according to Claim 7 in which the said eluted conjugate is then split to remove the said collagen-binding portion, and the said substance is then isolated.

[illegible]

Fig. 2.

**FIBRIN HEPARIN
S AUREUS**

COLLAGEN

DNA

STUDY 3

HEPARIN

FIBRIN

Fig. 3a.

FIRST SEQ. NO. =

1
S K R Q A Q Q M V O P Q S P V A V S O S K P G C Y D N G K H Y Q I N Q W E R
10 20 30 40 50 60 70 80 90 100 110 120
G A G A G C A G A G G C G T C A G C A G T G C C C G G T G G C T G T C A G C A T G S A A C C A G T A T C A G A T A A T A C A C A G T T G S A G C S

2
T Y L G N V L V C T C Y G G S R G F N C E S K P E A E E T C F D K Y T G N T Y R
130 140 150 160 170 180 190 200 210 220 230 240
G A C T A C C T A G T A N T G T T G T A T T G S A G A G C C G A G T T T A C T G C A A G T T A G C T G A G C T G T G C T T T G A C A G T A C T T G S A A C C A G T A C G

3
Y G D T Y E R P K D S M I W D C T C I G A G R G R I S C T I A N R C H E G G O S
250 260 270 280 290 300 310 320 330 340 350 360
A G T G G S T G A C A C T T A T G A G C T C T A A G A C T C A T C T G S A G C T A C T G C A T C G G G C T G G G C G A G G A A T A G C T G T A C C A T G C C A A A C C G C T G C C A T G A A G S G G T C A G T C

4
Y K I G D T W R R P H E T G G Y M L E C V C L G N G K G E V T C K P I A E K C F
370 380 390 400 410 420 430 440 450 460 470 480
C T A C A G A T T G S T G A C A C T G S A G A G C C A C A T G A G A C T G T T A G A T T A G A T T G S T G T T G T A T T G S A A A G S A A A T G S A C C T G C A A G C C A T A G C T T G A A G A T G T T T

5
D H A A G T S Y V V G E T V E K P Y Q G W M N V D C T C L G E G S G R I T C T S
490 500 510 520 530 540 550 560 570 580 590 600
T G A T C A T G C T G C A C T T A T G T G T C G A G A A A C S T G S A G A G C C T A C C A A G C T G S A T A T G T A G A T T G T A T T G C T T G S A G A A G S C A A G C C A G C C A T C A C T T G C A C T T C

6
R N R C N D Q D T R T S Y R I G D T W S K K D N R G N L L Q C I C T G N G R G E
610 620 630 640 650 660 670 680 690 700 710 720
T A G A A T A G A T S C A C G A T C A G A C A C A G S A C A C T T A T A G A T T T G A G A C A C T T G S A G A C A G S A T A T T G S A G A A A C T G C T C C A G T G C A T C T T G C A C A G C C A A G S G G C C A G A

7
W K C E R H T S V Q T T S S G S G P F T D V R A A V Y Q P Q P H P P P P P Y G H
730 740 750 760 770 780 790 800 810 820 830 840
G T G S A G T T G T G A G A G C A C A C T T G T G S A G A C A C A T G S A G S G A T C T G S G C C C T T C A G G S A T G T T G C A A A C C G C A G C C T C A G C C C C A G C C T C C T T C C T A T T G S C A

8
C V T D S G V V S Y G M Q V L K T Q G N K Q M L C T C L G N G V S C Q E T A Y
850 860 870 880 890 900 910 920 930 940 950 960
C T G T G T C A G A C A G A C T G T G T G T C T A C T G T G G G A T T G C A G T G T T G A G A C A C A G S A A A T A G C A A A T T C T T T G S A G T G C C T G S G C A A G S G A G T C A G C T T C C A A G A C A G A C T G T

9

Fig. 3c.

15														
I	K	L	K	P	G	V	V	Y	E	G	O	L	I	S
C	A	T	A	A	G	O	O	T	G	A	G	C	T	C
1930	1940	1950	1960	1970	1980	1990	2000	2010	2020	2030	2040			
16														
S	N	T	Y	T	G	E	T	T	P	F	S	P	L	V
C	A	G	A	A	C	O	O	S	T	G	A	G	A	G
2050	2060	2070	2080	2090	2100	2110	2120	2130	2140	2150	2160			
17														
S	G	F	R	V	E	E	L	S	E	G	D	E	P	Q
G	T	O	O	S	S	T	G	A	G	A	T	G	A	G
2170	2180	2190	2200	2210	2220	2230	2240	2250	2260	2270	2280			
18														
V	N	V	Y	Q	I	S	E	D	G	E	Q	S	L	I
T	G	T	A	T	C	A	G	A	T	C	A	G	A	T
2290	2300	2310	2320	2330	2340	2350	2360	2370	2380	2390	2400			
19														
V	V	R	W	S	R	P	Q	A	P	I	T	G	R	I
T	G	T	T	O	S	T	G	A	G	A	T	G	A	T
2410	2420	2430	2440	2450	2460	2470	2480	2490	2500	2510	2520			
20														
S	D	L	Q	P	G	V	Q	Y	N	I	T	I	A	V
C	A	G	T	G	C	A	G	T	G	A	T	G	A	T
2530	2540	2550	2560	2570	2580	2590	2600	2610	2620	2630	2640			
21														
P	S	R	D	L	Q	F	V	E	Y	T	O	V	K	Y
G	O	O	C	T	O	O	S	T	G	A	G	A	T	G
2650	2660	2670	2680	2690	2700	2710	2720	2730	2740	2750	2760			
22														
G	E	H	G	O	R	L	P	I	S	R	N	T	F	A
T	G	O	S	A	C	O	S	S	T	G	A	G	A	T
2770	2780	2790	2800	2810	2820	2830	2840	2850	2860	2870	2880			

Fig. 3h.

G K T Y H Y G E Q W O K E Y L G A I C S C T C F G G Q R G V R C D N C R R P G G ³⁶
 TGGAGAGCA TACCAGCTAGAGAGACAGGTGGCAAGAGATATCTGGGTGGCAATTGGCTTGGACATATGGCTTGGACATCTGGACAGCTGGGGGG
 6130 6740 6750 6760 6770 6780 6790 6800 6810 6820 6830 6840
 E P S P E G T T G Q S Y N Q Y S Q R Y H Q R T N T N V N C P I E C F M P L D V O
 TGAACCCAGCTGGCCAGAGCACTAGTGGCCAGTCTCAGAGATACCATGAGACACACACTAATGTAAATGGCCANTGGTGGCTTGAATGATGCA
 6850 6860 6870 6880 6890 6900 6910 6920 6930 6940 6950 6960
 A D R E D S R E
 GGCCTGACAGAGAGATTGGGAGAGTAATCATCTTCCATTCAGAGAGACAGCAATGTCTCTCTGGCAGATGCTATCTAACTGGAGTGTATGACAGAGCCAGCTTAGAGTTGTTC
 6970 6980 6990 7000 7010 7020 7030 7040 7050 7060 7070 7080
 TTCTTTCTTAAAGCCCTTTTGGCTGGAGAGGTTCCTGAGCTTCAGCTCACTCAAGCTTCTGAGCATCAGCTTGGAGTTTCTGAGGGTTTCTGATTAATGAGGGCTGGCAGATT
 7090 7100 7110 7120 7130 7140 7150 7160 7170 7180 7190 7200
 GCGCTGTCTGGCTGGAGATTTCAATAGCCCTCAGATATTTAAATGAGTGTATCTTAGATTTGGTTTGGGATCATAGAGAAAGCATATGCGAGCCAGCCAGATGCAATGTTTGGAAAT
 7210 7220 7230 7240 7250 7260 7270 7280 7290 7300 7310 7320
 GATATGACCAAAATTTTAAATGAGAAAGTCAACCAAGAGCTTCTGCTTCACTTAAGTGTCTGGCCGCAANTACTGTGAGAGACAGCATGATCTGTATCTGTGTATATTTAAATATGCA
 7330 7340 7350 7360 7370 7380 7390 7400 7410 7420 7430 7440
 CAGTACTGACTTTTCCAAATGATCTAGTAATGGCTAGAAATATCTTCTCTTACTGTATTTATCTAATTTTCCAGATTTTTTAATGCGAAAAATTTGATATGGAAGAGCTTAGT
 7450 7460 7470 7480 7490 7500 7510 7520 7530 7540 7550 7560
 ATGCGATTGATAGAGCAATTGGTATTAATATGGTGGGTGATATTTTATTTACTGTATGTGGCAAGCTTACTACTGTGGAGAGCAACTGTGTTTAAATGAAGATTTGATATGCA
 7570 7580 7590 7600 7610 7620 7630 7640 7650 7660 7670 7680
 AAAAAAAAAAAAAAAAAAAAAA
 7690 7700 5 15 25 35 45 55 65 75 85 95

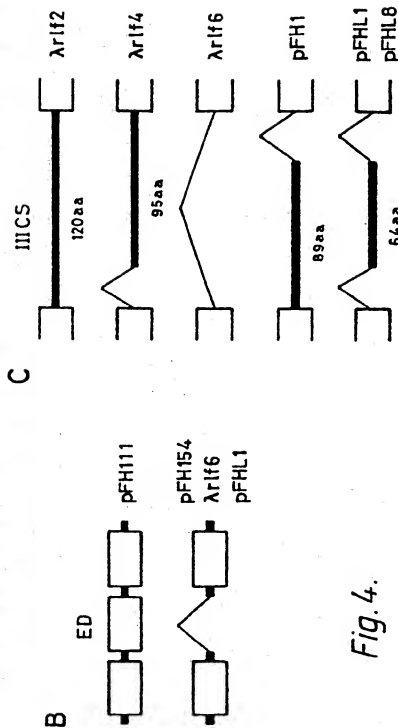
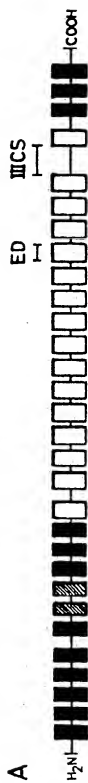


Fig. 4.



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020775
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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IN C I 4)
X	WO - A1 - 84/00 540 (LA JOLLA CANCER RESEARCH FOUNDATION) * Claims 1,10,13,22 *	1	C 07 K 15/14 C 12 N 15/00 C 07 H 21/04 C 12 P 21/00
D, A	ANNUAL REVIEW OF BIOCHEMISTRY, vol. 52, 1983, (Palo Alto, USA) K.M. YAMADA "Cell Surface Interactions with Extracellular Materials" pages 761-799 * Pages 768-781 *	1	C 12 N 1/20 A 61 K 37/02 C 12 N 1/20; C 12 R 1:19)
D, A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 80, no. 11, June 1983 (Baltimore, USA) A.R. KORNBLIHTT et al. "Isolation and characterization of cDNA clones for human and bovine fibronectins" pages 3218-3222 * Totality *	1, 9	
			TECHNICAL FIELDS SEARCHED (IN C I 4)
			C 07 K C 12 N C 07 H C 12 P
D, A	CELL, vol. 35, no. 2, December 1983 (Part. 1) (Cambridge, Mass, USA) J.E. SCHWARZBAUER et al. "Three Different Fibronectin mRNAs Arise by Alternative Splicing within the Coding Region" pages 421-431 * Totality *	1, 9	
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 06-10-1986	Examiner WOLF
CATEGORY OF CITED DOCUMENTS			
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0207751

- 2 -
Application number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 86304998.7
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	EP - A2 - 0 114 685 (THE GREEN CROSS CORPORATION) * Pages 1-5 *	1,6,7	
A	EP - A2 - 0 022 052 (AMRANI) * Pages 1-5 *	1,6,7	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
Place of search VIENNA		Date of completion of the search 06-10-1986	Examiner WOLF
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X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document	

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